Current Topics in Biophysics,2013, 36, 7-13 DOI: 10.2478/ctb-2013-0006

EPR STUDY OF PARAMAGNETIC CENTERS IN HUMAN BLOOD*

TOMASZ KUBIAK, RYSZARD KRZYMINIEWSKI, BERNADETA DOBOSZ

Medical Physics Division, Faculty of Physics, Adam Mickiewicz University, Poznań, Poland

Received November 14, 2013; accepted November 25, 2013; published online November 28, 2013

Electron paramagnetic resonance investigations of paramagnetic centers in whole human blood were carried out at 170 K using X-band EPR spectrometer. The study included a group of patients and healthy volunteers. The EPR signals from high spin Fe^{3+} ions in transferrin (g = 4.2) and Cu^{2+} ions in ceruloplasmin (g = 2.05) are characteristic of each frozen blood sample. An overview of all recorded spectra revealed in several cases additional lines derived from high spin Fe^{3+} ions in methemoglobin (g = 5.8 - 6), free radicals (g = 2.002 - 2.005) and various low spin ferriheme complexes (g = 2.21 - 2.91). The lines from cytochromes (g = 3.03 and 3.27) were observed only twice. The EPR measurements have not confirmed the correlation between the occurrence of a particular type of low-spin iron complex and a specific disease entity. Moreover, the presence of EPR lines from trivalent iron also did not differentiate patients from healthy volunteers.

INTRODUCTION

Paramagnetic centers in human blood include primarily the molecular complexes containing iron Fe^{3+} (transferrin, methemoglobin) or copper Cu^{2+} ions (ceruloplasmin) and free radicals.

Human ceruloplasmin (hCP) is a glycoprotein with a molecular weight of 132 kDa (Zaitseva, Zaitsev, Card, Moshkov, Bax, Ralph & Lindley, 1996; Bento, Peixoto, Zaitsev & Lindley, 2007; Farver, Bendahl, Skov & Pecht, 1999) and the carbohydrate content between 7 and 8% (Bento et al., 2007). The concentration of ceruloplasmin in the blood plasma of healthy adult people amounts to 300 mg/ml (Healy & Tipton, 2007). The hCP protein is comprised of six domains and contains six copper ions. Three of them form a trinuclear cluster at the interface of domains 1 and 6 and the other three are arranged individually in domains 2, 4 and 6 (Zaitseva et al., 1996). Paramagnetic ions (Cu type I) in mononuclear sites have more complex EPR spectrum than more regularly coordinated paramagnetic ion from the cluster (Cu type II). Two remaining antiferromagnetically coupled ions from cluster (Cu type III) are EPR silent (Healy & Tipton, 2007). In EPR spectra of the purified human ceruloplasmin one can distinguish the signal from Cu type II with $g_{\|}$= 2.25 and $A_{\|}$= 18 mT and two dissimilar signals from Cu type I centers with different values of the hyperfine splitting constant ($A_{\|}$= 7.2 mT and $A_{\|}$= 9 mT), but similar g values ($g_{\|}$= 2.20 and 2.21) (Kouoh Elombo, Radosevich, Poulle, Descamps, Chtourou, Burnouf, Catteau, Bernier & Cotelle, 2000). The study of copper sites is important in context of an understanding of the role of the protein in human organism.

Ceruloplasmin is the acute phase reactant and the multifunctional enzyme, which shows amino-oxidase, superoxide dismutase and ferro-oxidase activity (Senra Varela, Bosco Lopez Saez & Quintela Senra, 1997). It catalyzes the oxidization of Fe^{2+} to Fe^{3+}, which is essential to load apotransferrin with iron and also the reoxidation of Cu^{+} to Cu^{2+}. Ceruloplasmin is synthesized in the liver, but may also be produced by cancer cells. In case of malignant tumors the level of copper in the plasma (Zowczak, Iskra, Torliński & Cofta, 2001) and the concentration of ceruloplasmin increase (Senra Varela et al., 1997; Özyilkan, Baltali, Özyilkan, Tekuzman, Kars & Firat 1992) and thus also increases the rate of synthesis and secretion of this glycoprotein by the liver. Tumor cells can capture a non-ceruloplasmin copper from plasma, so that they contain a relatively large amount of copper (Zowczak et al., 2001). Patients with head and neck cancer had significantly elevated serum ceruloplasmin level as compared to the control group and this increase was directly proportional to the stage of cancer. A significant decrease in ceruloplasmin level was observed after radiotherapy, but the value was still higher than in the control group (Sachdeva, Girdhar, Gulati & Lal, 1993).

* This paper was presented at IX International Workshop on EPR (ESR) in Biology and Medicine, 7-10 October, 2013, Kraków, Poland.

Human transferrin (Tf) fulfils a lot of functions. Although the primary role of this protein is to transport and deliver iron to cells, it is also involved in the processes of growth, proliferation, differentiation and apoptosis of cells (Gomme & McCann, 2005). Two iron ions can be easily bound to apotransferrin molecule provided that they are found in the ferric form. Therefore, the oxidation of Fe^{2+} to Fe^{3+} by ceruloplasmin is necessary to load apotransferrin with iron. Under normal conditions only about 30% of transferrin binding sites are occupied by iron ions (Sanna, Garribba & Micera, 2009). Apotransferrin is EPR-silent, but when it bounds Fe^{3+} ions one can observe a distinct EPR signal from high-spin (S = 5/2) ferric non-haem iron in transferrin (Dunne, Caron, Menu, Alayash, Buehler, Wilson, Silaghi-Dumitrescu, Faivre & Cooper, 2006). Transferrin constitutes a part of the defense system against the excess of free iron in the organism. The presence of iron unbound to proteins leads, via the so-called Fenton reaction, to the formation of highly reactive hydroxyl radicals, which cause damages to lipids and DNA (Liehr & Jones, 2001). Postmenopausal women, in whom a cessation of menstruation reduces the loss of iron, are exposed to the body overload with this element and accelerated production of reactive oxygen species, which strengthen the process of carcinogenesis (Jian, Yang, Dai, Eckard, Axelrod, Smith & Huang, 2011; Kubiak, 2013).

The EPR studies of human blood revealed also the anisotropic signal ($g_{\perp}$ = 6 and $g_{\parallel}$ = 2) from high-spin Fe^{3+} in methemoglobin (Svistunenko, Dunne, Fryer, Nicholls, Reeder, Wilson, Bigotti, Cutruzzola & Cooper, 2002; Pulatova, Sharygin, Shlyakova, Sipyagina & Wasserman, 2009). Both oxyhemoglobin and deoxyhemoglobin under the influence of different factors might turn into methemoglobin and a healthy man always has got the small amount of this protein in blood. The higher concentration of metHb (> 1.5 g/dL) causes methemoglobinemia, which is accompanied by cyanosis (Hamirani, Franklin, Grifka & Stainback, 2008). Furthermore, at least two low spin forms (with two pairs of electrons coupled and a total spin of 1/2) of metHb are present at low temperature, including one with a g_x = 2.8 and the other with g_x = 2.98 (bis-histidine state) (Svistunenko, Sharpe, Nicholls, Blenkinsop, Davies, Dunne, Wilson & Cooper, 2000).

Free radicals in blood can be produced in the reaction of hydrogen peroxide with human methemoglobin (Svistunenko et al., 2002). Peroxyl radical (with g = 2.033) is derived from tryptophan residues, while nonperoxyl (with g = 2.005) was identified as tyrosyl radical (Svistunenko et al., 2002; Dunne et al., 2006). Plasma ascorbate has the ability to scavenge globin free radicals. The reduction of these species by ascorbate entails the formation of less reactive ascorbyl radical. The increase in the concentration of ascorbyl radicals reflects the rate of the one-electron oxidation of ascorbate and indicates oxidative challenges, also those caused by iron overload (Vasquez-Vivar, Santos, Junqueira & Augusto, 1996). The EPR spectrum of ascorbyl radical is a doublet at room temperature and a narrow singlet (ΔB_{pp} = 0.9 mT) at 10 K (Dunne et al., 2006). In blood at 77K the line has g = 2.0057 (Pulatova et al., 2009). Human erythrocytes efficiently recycle ascorbate, because ascorbyl free radicals are reduced by the NADH- and NADPH- dependent reductases (May, Qu & Cobb, 2004). It is worth noting that adrenaline radicals (singlet, ΔB_{pp} = 1.4 mT, g = 2.003) and adrenochrome radicals (singlet ΔB_{pp} = 0.9 mT, g = 2.004) were also detected in the blood (Pulatova et al., 2009).

The aim of the present work is to investigate, by means of EPR spectroscopy, paramagnetic centers present in whole blood of cancer patients and to determine whether some centers are characteristic of a specific disease entity and do not occur in the blood of healthy volunteers.

MATERIALS AND METHODS

EPR investigations of whole human blood have been being carried out in the Medical Physics Division at Adam Mickiewicz University in Poznan for several years. The studied material consists of samples from patients with various types of cancer (for example breast cancer). Blood was also drawn from healthy volunteers. All specimens were placed in liquid nitrogen immediately after collection and stored in deep freeze in dewar flask.

The EPR measurements were carried out at 170 K using X-band (9.4 GHz) spectrometer Bruker EPR EMX-10 equipped with a digital temperature control unit ER 4131VT. A standard rectangular cavity ER 4102ST was used in the experiment. During the low temperature measurements a purging nitrogen gas flow through the cavity was used to avoid water condensation inside the cavity. The following settings were used: microwave power 20 mW, modulation frequency 100 kHz, second modulation amplitude 1 mT. Each sample was measured in three sweep ranges: 650 mT (centre 335 mT) and 50 mT (centers respectively in 335 mT and 160 mT). To minimize the noise the spectra were accumulated 9 or 25 times. The EPR signals recorded in sweep range 650 mT were smoothed using adjacent averaging method in order to increase the visibility of the weak lines. All signals acquired in sweep range 50 mT were filtered using a low-pass filter. The cutoff frequency was chosen so that the signal would not be distorted.

RESULTS AND DISCUSSION

At 170K in EPR spectra of frozen human blood appears the signal, which consists of three distinct components with different amplitudes (Fig.1). This signal is present in all cases (both in healthy and ill people). The *g* factor, assigned to the line formed by the merger of two components with highest amplitudes, is in the range of g = 4.14 - 4.16. Comparison with the literature shows that this signal originates from Fe^{3+} in transferrin (g = 4.2) (Hirota, Haida, Mohtarami, Takeda, Iwamoto, Shioya, Tsuji, Hasumi, Nakazawa, 2000; Pocklington & Foster, 1977), which is also confirmed by the very characteristic shape. The first component (counting in the direction of increasing values of magnetic field induction), for which g = 4.30 - 4.37, has the lowest amplitude and the narrowest line width. The amplitude of the second component with g = 4.23 - 4.27 is greater than the amplitude of the first one. The third component, with g = 4.11 - 4.13, has got the highest amplitude and the greatest line width.

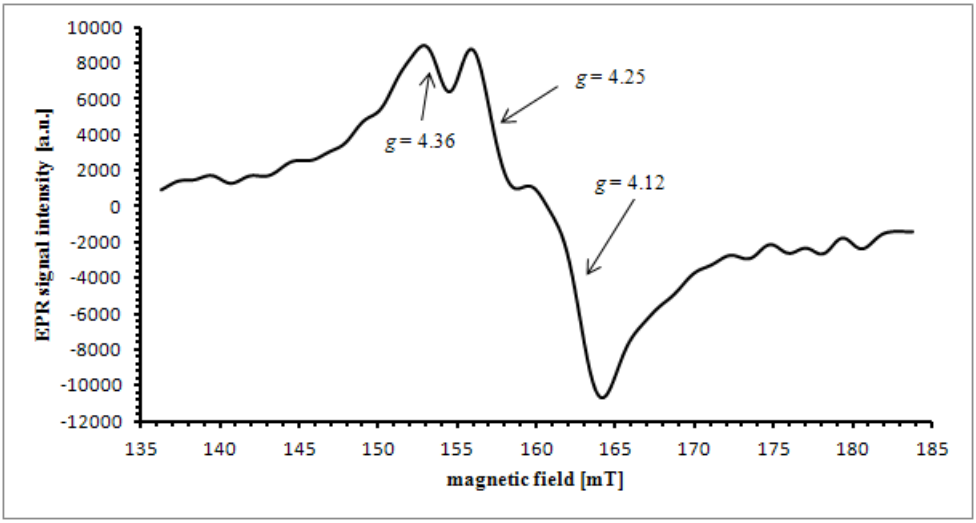

Fig. 1. Typical EPR signal from Fe^{3+} ions in transferrin in whole human blood consists of three components. Spectrum recorded at 170 K.

The literature values of *g* factors for three components are $g \approx 4.39$; $g \approx 4.19$ and $g \approx 4.07$, respectively (Rottman, Doi, Zak, Aasa & Aisen, 1989). Two non-heme Fe^{3+} ions are in the high-spin state S = 5/2 and their EPR spectrum is characteristic of the compounds in which iron is present in the rhombic symmetry system (Krzyminiewski, Kruczyński, Dobosz, Zając, Mackiewicz, Leporowska & Folwaczna, 2011; Preoteasa,

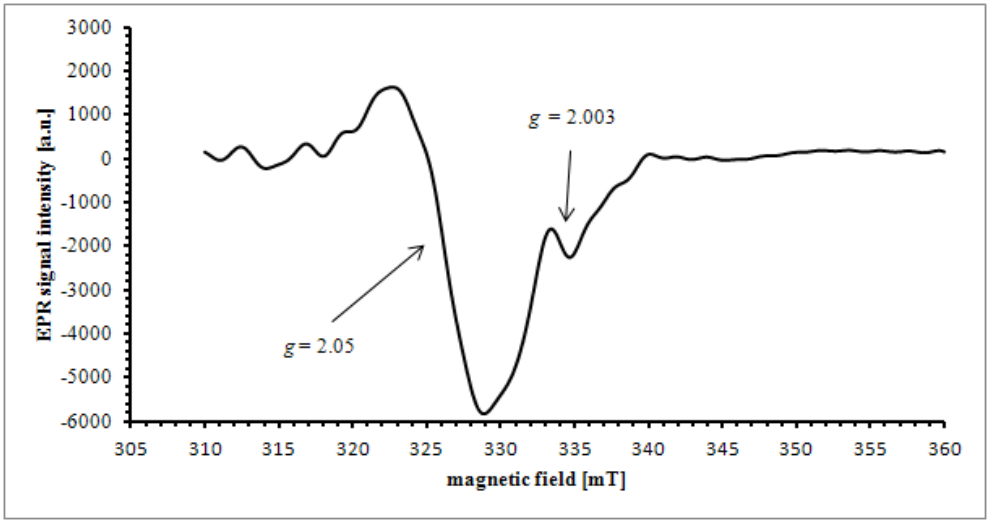

Fig. 2. The EPR signal from Cu^{2+} ions in ceruloplasmin (g = 2.05) and the line from free radical (g = 2.003). The spectrum of whole blood of 68-year-old female breast cancer patient was recorded at 170K.

Schianchi, Camillo Giori, Duliu, Butturini & Izzi, 2013). In transferrin each iron atom is bound in a distorted octahedral coordination to four amino acid residues (two tyrosines, one histidine, and an aspartic acid) and two oxygen atoms from a synergistically bound carbonate ion (Adams, Mason, He, Halbrooks, Briggs, Smith, MacGillivray & Everse, 2003).

In the EPR spectra of whole blood occurs also the line for which g = 2.05 (Fig.2). This corresponds to the literature value: g = 2.049 assigned to Cu^{2+} ions in ceruloplasmin (Hirota et al., 2000; Pocklington & Foster 1977; Foster, Pocklington, Miller & Mallard, 1973). This is perpendicular hyperfine line of the Cu^{2+} signal (Kleinhans, Kline, Dugan & Williams 1983), most intensive component, which comes from the Cu type II (Kouoh Elombo et al., 2000). Peak-to-peak widths of the recorded signals of Cu^{2+} from ceruloplasmin are in the range of 5.3 - 7.5 mT. Cu type II signal is characteristic of tetragonally coordinated copper complex (Kouoh Elombo et al., 2000). It is worth noting that the line from ceruloplasmin appears both in ill and healthy people.

In Fig.2 there is small signal with g = 2.003 and peak-to-peak width 1.27 mT. Generally, the lines with g factors in the range g = 2.002 - 2.005 and ΔB_{pp} = 1.03 - 1.37 mT were observed in EPR spectra of whole human blood of ≈ 30% cancer patients. These signals probably comes from free radicals.

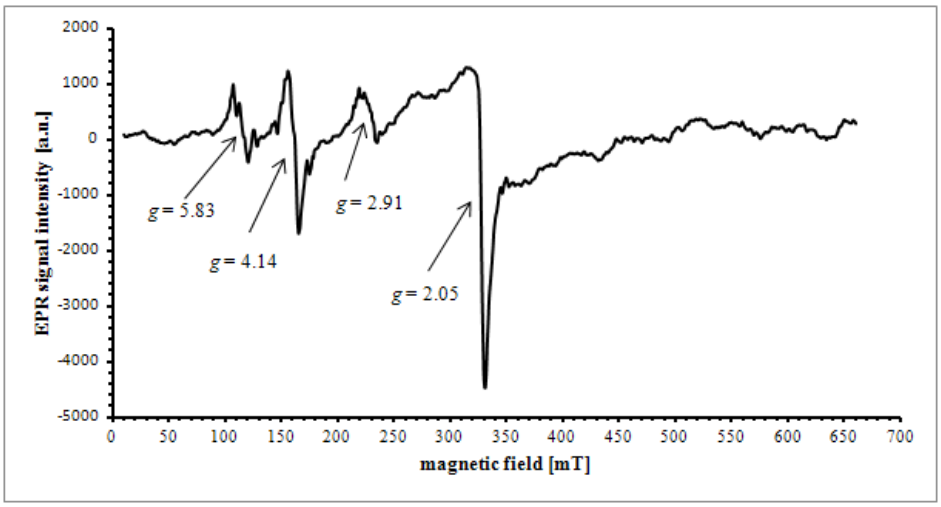

Fig. 3. EPR spectrum of whole blood of 68-year-old female breast cancer patient contains the signals from: Cu^{2+} in ceruloplasmin (g = 2.05), high spin Fe^{3+} in transferrin (g = 4.14), high spin Fe^{3+} in methemoglobin (g = 5.83) and low-spin ferriheme complex (g = 2.91). Spectrum recorded at 170K.

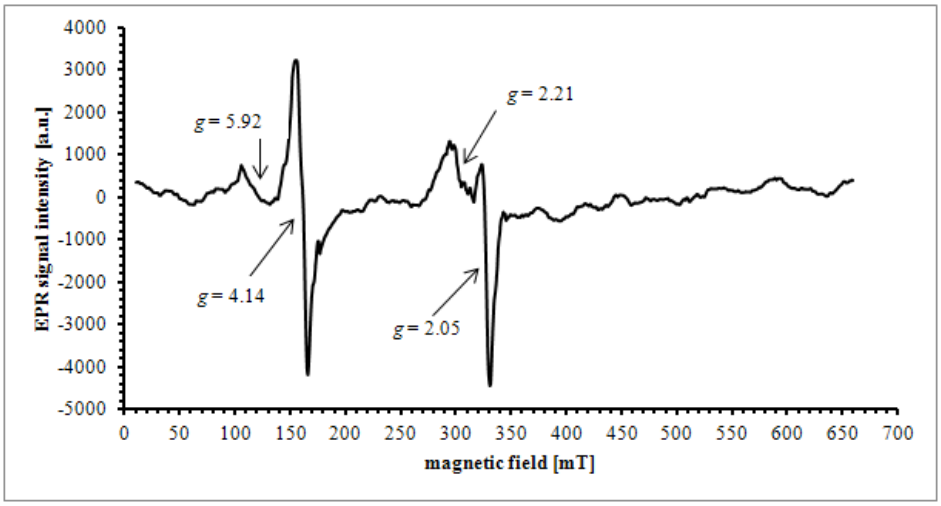

Fig. 4. EPR spectrum of whole blood of 51-year-old female breast cancer patient contains the signals from: Cu^{2+} in ceruloplasmin (g = 2.05), high spin Fe^{3+} in transferrin (g = 4.14), high spin Fe^{3+} in methemoglobin (g = 5.92) and low-spin ferriheme complex (g = 2.21). Spectrum recorded at 170K.

In some spectra appears the signal with g = 5.83 - 6. (Fig.3-6). It can be attributed to high-spin Fe^{3+} in methemoglobin (Preoteasa et. al., 2013). In Fig.3-4 is visible that the line from metHb indicates the presence of two superimposed signals. They can originate from iron ions in α and β subunits in metHb (it was observed a rhombic distortion in the almost axial (tetragonal) symmetry of iron in two subunits) or result from two different conformations of this protein, which is less probably (Svistunenko et al., 2000).

In some cases one can also distinguish EPR signals from different Fe^{3+} complexes. In Fig.3 there is the line with g = 2.91, in Fig.4 and Fig.6 with g = 2.21, in Fig.5 lines with g = 2.26 and g = 2.56.

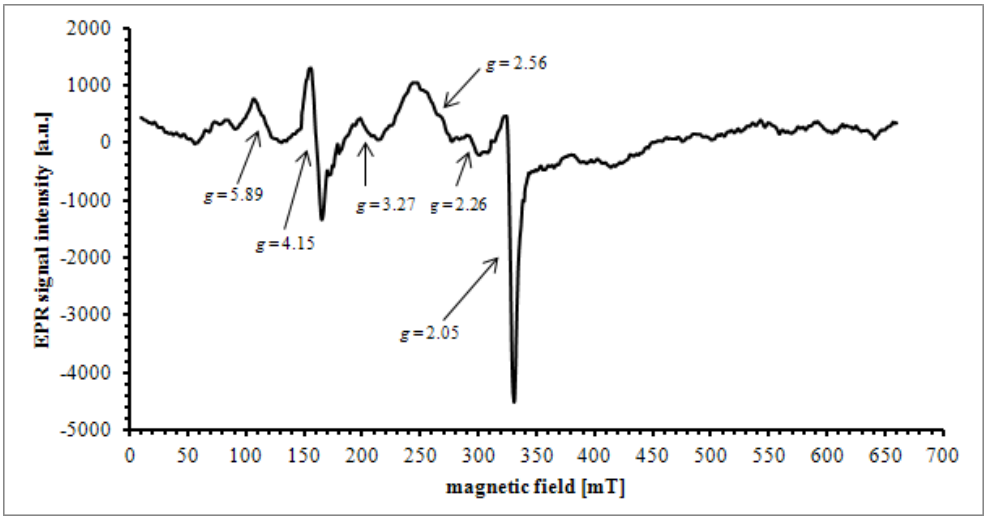

Fig. 5. EPR spectrum of whole blood of another 51-year-old female breast cancer patient contains the signals from: Cu^{2+} in ceruloplasmin (g = 2.05), high spin Fe^{3+} in transferrin (g = 4.15), high spin Fe^{3+} in methemoglobin (g = 5.89), low-spin ferriheme complexes (g = 2.26 and 2.56) and cytochrome (g = 3.27). Spectrum recorded at 170K.

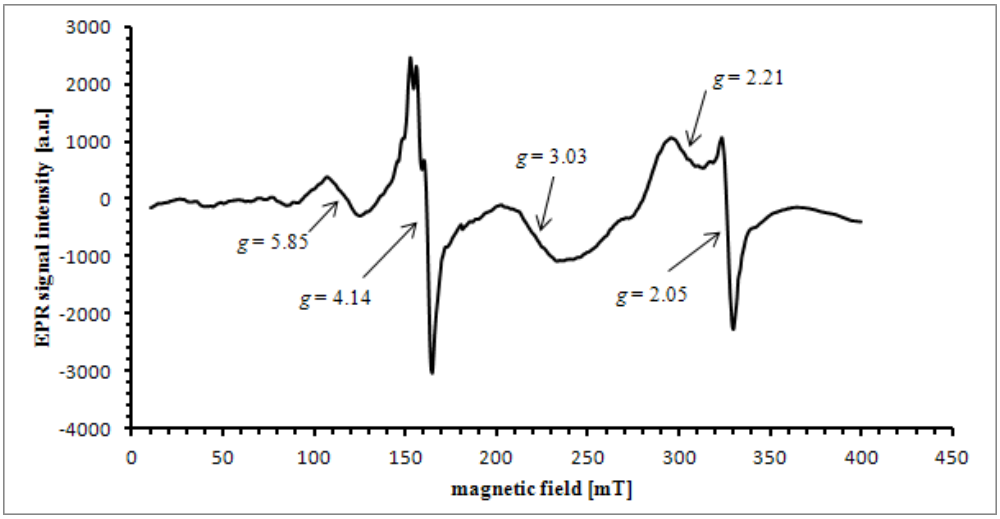

Fig. 6. EPR spectrum of whole blood of healthy person contains the signals from: Cu^{2+} in ceruloplasmin (g = 2.05), high spin Fe^{3+} in transferrin (g = 4.14), high spin Fe^{3+} in methemoglobin (g = 5.85), low-spin ferriheme complex (g = 2.21) and cytochrome (g = 3.03). Spectrum recorded at 170K.

These signals may be due to hemine complexes (hydroxy form of MetHb at alkaline pH and hydroxy form of free α chains of HbA) or hemichromes reversibly formed from HbA (Preoteasa et al., 2013), but their precise identification is difficult. Generally, the resonance lines at g = 2.15 - 2.30 are typical of low-spin (S = 1/2) ferriheme proteins and the lines at g > 3 are characteristic of various cytochromes (Preoteasa et al., 2013). Signals from cytochromes were detected only twice. The line with g = 3.27 was found in EPR

spectrum of whole blood of 51-year-old female breast cancer patient (Fig.5) and the signal with g = 3.03 (Fig.6) in spectrum of whole blood of healthy person.

CONCLUSION

The EPR study showed that the signals from high spin Fe^{3+} ions in transferrin (g = 4.2) and Cu^{2+} ions in ceruloplasmin (g = 2.05) are present in the spectra of frozen whole blood both in healthy people and patients suffering from cancer. EPR lines derived from high spin Fe^{3+} ions in methemoglobin (g = 5.8 - 6) and various low spin ferriheme complexes (g = 2.21 - 2.91) were detected in some patients and also in several healthy volunteers. Therefore, it cannot be unequivocally established whether the presence of EPR line from specific trivalent iron complex is characteristic of particular disease entity and allows to differentiate patients from healthy people.

ACKNOWLEDGMENTS

Special thanks to everyone who contributed to the process of collecting blood samples from patients.

REFERENCES

Adams T. E., Mason A. B., He Q. Y., Halbrooks P. J., Briggs S. K., Smith V. C., MacGillivray R. T. A. & Everse S. J. (2003). The Position of Arginine 124 Controls the Rate of Iron Release from the N- lobe of Human Serum Transferrin, *J. Biol. Chem*. **278(8)**, 6027-6033.

Bento I., Peixoto C., Zaitsev V. N. & Lindley P. F. (2007). Ceruloplasmin revisited: structural and functional roles of various metal cation- binding sites. *Acta Cryst. D* **63**, 240-248.

Dunne J., Caron A., Menu P., Alayash A. J, Buehler P. W., Wilson M. T., Silaghi-Dumitrescu R., Faivre B. & Cooper C. E. (2006). Ascorbate removes key precursors to oxidative damage by cell-free haemoglobinin vitro and in vivo, *Biochem. J*. **399**, 513-524.

Farver O., Bendahl L., Skov L. K. & Pecht I. (1999). Human Ceruloplasmin. Intramolecular electron transfer kinetics and equilibration. *J. Biol. Chem.* **274**, 26135-26140.

Foster M. A., Pocklington T., Miller J. D. B. & Mallard J. R (1973). A study of electron spin resonance spectra of whole blood from normal and tumour bearing patients. *Br. J. Cancer* **28**, 340-348.

Gomme P. T. & McCann K. B. (2005). Transferrin: structure, function and potential therapeutic actions. *Drug Discov. Today* **10**, 267-273.

Hamirani Y. S., Franklin W., Grifka R. G. & Stainback R. F. 2008. Methemoglobinemia in a Young Man. *Tex. Heart Inst. J*. **35(1)**, 76-77.

Healy J. & Tipton K. (2007). Ceruloplasmin and what it might do. *J. Neural. Transm*. **114**, 777-781.

Hirota Y., Haida M., Mohtarami F., Takeda K., Iwamoto T., Shioya S., Tsuji C., Hasumi K. & Nakazawa H. (2000). Implication of ESR signals from ceruloplasmin (Cu2+) and transferrin (Fe3+) in pleural effusion of lung diseases. *Pathophysiology* **7**, 41-45.

Jian J., Yang Q., Dai J., Eckard J., Axelrod D., Smith J. & Huang X. (2011). Effects of iron deficiency and iron overload on angiogenesis and oxidative stress - a potential dual role for iron in breast cancer. Free Radic. Biol. Med. **50**, 841-847.

Kleinhans F. W., Kline S. C., Dugan W. M. Jr. & Williams J. G. (1983). Comparison of Electron Paramagnetic Resonance and Atomic Absorption Serum Copper Measurements in Human Normal Control and Cancer Patient, *Cancer Res.* **43(7)**, 3447-3450.

Kouoh Elombo F., Radosevich M., Poulle M., Descamps J., Chtourou S., Burnouf T., Catteau J. P., Bernier J. L. & Cotelle N. (2000). Purification of human ceruloplasmin as a by- product of C1-inhibitor. *Biol. Pharm. Bull*. **23**, 1406-1409.

Krzyminiewski R., Kruczyński Z., Dobosz B., Zając A., Mackiewicz A., Leporowska E. & Folwaczna S. (2011). EPR Study of Iron Ion Complexes in Human Blood. *Appl. Magn. Reson*. **40**, 321-330.

Kubiak T. (2013). Correlation between iron metabolism and development of breast cancer in pre- and postmenopausal women. *Prz. Menopauz*. **4**, 339-342. [Article in Polish]

Liehr J. G. & Jones J. S. (2001). Role of iron in estrogen-induced cancer. *Curr. Med. Chem*. **8**, 839-849.

May J. M., Qu Z. & Cobb C. E. (2004). Human Erythrocyte Recycling of Ascorbic Acid. *J. Biol. Chem*. **279(15)**, 14975-14982.

Özyilkan Ö., Baltali E., Özyilkan E., Tekuzman G., Kars A. & Firat D. (1992). Ceruloplasmin level in women with breast disease. *Acta Oncol.* **31**, 843-846.

Pocklington T & Foster M. A. (1977). Electron spin resonance of ceruloplasmin and iron transferrin in blood of patients with various malignant diseases. *Br. J. Cancer* **36**, 369-374.

Preoteasa E. A., Schianchi G., Camillo Giori D., Duliu O. G., Butturini A. & Izzi G. (2013). Unexpected Detection of Low- and High-Spin Ferrihemoglobin Derivatives in Blood Serum of Polytransfused Patients with Homozygous - Thalassemia under Chelation Therapy. An EPR Study. *Dig. J. Nanomater. Bios.* **8(2)**, 469-499.

Pulatova M. K., Sharygin V. L., Shlyakova T. G., Sipyagina E. A. & Wasserman A. M. (2009). Use of EPR Spectroscopy to Check the Changes in Organism Radioresistance. Clinical Results. *Biophysics*, **54(2)**, 223-231.

Rottman G.A., Doi K., Zak O., Aasa R. & Aisen P. (1989). Hyperfine Interactions of ^{57}Fe in Human Transferrin: An ENDOR Spectroscopic Study. *J. Am. Chem. Soc*. **111**, 8613-8618.

Sachdeva O.P., Girdhar V., Gulati S. P. & Lal H. (1993). Serum ceruloplasmin levels in head and neck cancers *Indian. J. Clin. Biochem*. **8**, 51-53.

Sanna D., Garribba E. & Micera G. (2009). Interaction of VO^{2+} ion with human serum transferrin and albumin. *J. Inorg. Biochem*. **103**, 648-655.

Senra Varela A., Bosco Lopez Saez J. J. & Quintela Senra D. (1997). Serum ceruloplasmin as a diagnostic marker of cancer. *Cancer Lett.* **121**, 139-145.

Svistunenko D. A., Dunne J., Fryer M., Nicholls P., Reeder B.J., Wilson M. T, Bigotti M. G., Cutruzzola F. & Cooper C. E. (2002). Comparative Study of Tyrosine Radicals in Hemoglobin and Myoglobins Treated with Hydrogen Peroxide, *Biophys. J.* **83,** 2845-2855.

Svistunenko D. A., Sharpe M. A., Nicholls P., Blenkinsop C., Davies N. A., Dunne J., Wilson M. T. & Cooper C. E. (2000). The pH dependence of naturally occurring low-spin forms of methaemoglobin and metmyoglobin: an EPR study, *Biochem. J.* **351**, 595-605.

Vasquez-Vivar J., Santos A. M., Junqueira V. B. C. & Augusto O. (1996). Peroxynitrite-mediated formation of free radicals in human plasma: EPR detection of ascorbyl, albumin-thiyl and uric acid-derived free radicals, *Biochem. J.* **314**, 869-876.

Zaitseva I., Zaitsev V., Card G., Moshkov K., Bax B., Ralph A. & Lindley P. (1996). The X-ray structure of human serum ceruloplasmin at 3.1 Å: nature of the copper centres. *J. Biol. Inorg. Chem*. **1**, 15-23.

Zowczak M., Iskra M., Torliński L. & Cofta S. (2001). Analysis of Serum Copper and Zinc Concentrations in Cancer Patients. *Biol. Trace Elem. Res.* **82(1-3)**, 1-8.